ABSTRACT

The mixed epidermal growth factor receptor (EGFR)-DNA targeting properties of SMA41, a 5-(3-methyl-1,2,3-triazene-1-yl)-4-anilinoquinazoline designed to release N4-m-tolyl-quinazoline-4,6-diamine henceforth referred to as SMA52 [an inhibitor of EGFR tyrosine kinase (TK)] and methylidiazonium (a DNA methylating species) were studied in the O6-methylguanine-DNA methyltransferase (MGMT)-proficient and high EGFR-expressing epidermoid carcinoma of the vulva cell line A431. The effects of SMA41 were compared with those of SMA52 alone, and temozolomide (TEM), a clinical prodrug of 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MITC) that is inactive in MGMT-proficient cells. The results showed that 1) the chimeric SMA41 could degrade in serum-containing medium (t1/2 of ~30 min) to generate, as predicted, the free inhibitor SMA52 as the most abundant metabolite (~81% yield); 2) in contrast to SMA52 alone, the chimeric SMA41 and TEM induced significant DNA damage in A431 cells after 30-min or 2-h drug exposures, as confirmed by alkaline single-cell gel microelectrophoresis (comet) assay; 3) SMA41 showed 5-fold greater affinity for the ATP binding site of EGFR than independently synthesized SMA52 in an enzyme assay and blocked EGF-induced tyrosine phosphorylation and EGFR autophosphorylation in A431 cells in a dose-dependent manner; 4) these mixed targeting properties of SMA41, combined with its ability to be converted to another potent EGFR TK inhibitor (e.g., SMA52) by hydrolytic cleavage, translated into over 8-fold greater antiproliferative activity than TEM, which showed no EGFR targeting properties (IC50 competitive binding >100 μM); 5) under continuous drug exposure (3–6-day sulfonrophamidate and clonogenic assays), SMA41 was almost equipotent with SMA52; however, in a short 2-h drug exposure followed by incubation in drug-free media, SMA52 showed an almost complete loss of antiproliferative activity over the whole dose range. In contrast, SMA41 retained almost 100% of its activity, indicating a more sustained growth inhibitory activity. The results in toto suggest that the superior antiproliferative activity of SMA41 may be due to a combination of events associated with its binary EGFR TK and DNA targeting properties.

Over the past 20 years, acquired resistance mediated by DNA repair enzymes has often imposed severe limitations on the use of DNA-interactive agents and in many cases useful clinical antitumor activity could only be observed with the administration of multiple antitumor drugs of different mechanisms of action. Based upon this observation, we surmised that novel compounds with multiple intracellular targets would be more effective against resistant tumors than their classical counterparts. In this article, we describe the first attempt to combine two major mechanisms of action (inhibition of tyrosine kinase-mediated signaling and DNA targeting) into one single molecule (e.g., SMA41). The biochemical responses to the latter molecule were studied in the resistant Mer+ DNA repair-proficient human epidermoid carcinoma of the vulva cell line A431, which coexpresses high levels of EGFR TK.

The overexpression and dysfunction of TKs, directly or indirectly implicated in mitogenic signaling in tumor cells, have been extensively studied and are now considered the major functional differences between normal and tumor cells (Tsai et al., 1993; Sinha et al., 1995; Kondapaka and Reddy, 1996; Alaoui-Jamali et al., 1997; Sherwood et al., 1999). Because of their significant involvement in tumor progression, overexpressed receptor TKs have now become the modern targets for drug design and selective chemotherapeutic interventions (Levitzki and Gazit, 1995; Carroll et al., 1997;
Deininger et al., 1997). One such target is EGFR, which, in many patients, is associated with aggressive tumor progression and invasion (Turner et al., 1996; Moyer et al., 1997; Modjtabahi and Dean, 1998; Xie et al., 1999). It has already been demonstrated that blocking signal transduction mediated by the TK activity of EGFR translates into significant antitumor activity both in vitro and in vivo, and two novel agents are now in phase II clinical trials (Ward et al., 1994; Levitzki and Gazit, 1995; Rewcastle et al., 1995, 1997, 1998; Lanzè et al., 1997; Moyer et al., 1997). Despite being significantly less toxic than previous cytotoxic agents, in tumors where they cannot induce apoptosis, most TK inhibitors currently in clinical trial present the disadvantage of being cytostatic agents that induce reversible growth inhibitory activity (Smaill et al., 1999).

The disadvantages associated with both the classical cytotoxic agents and the modern RTK inhibitors, as well as the need for novel targets to circumvent DNA repair-associated chemoresistance, stimulated our interest in designing molecules with mixed EGFR tyrosine kinase and DNA targeting properties.

SMA41 exhibits two distinct structural characteristics: 1) a 1,2,3-triazene linkage, the pharmacophore of the active metabolites of dacarbazine or TEM; and 2) a 4-anilinoquinazoline moiety, the pharmacophore of the potent quinazoline class of EGFR TK inhibitors, which are now in clinical trial (Ching et al., 1993; Moyer et al., 1997).

The 3-alkyl-1,2,3-triazenes such as TEM, or its metabolite MTIC, are known to heterolyze to an aromatic amine (e.g., 5-aminoimidazole-4-carboxamide, Scheme 1) and an alkylidiazonium species (e.g., methylidiazonium, Scheme 1) under hydrolytic conditions (Cameron et al., 1985; Gibson et al., 1986; Baig and Stevens, 1987). Their mechanism of action is primarily based upon the generation of alkylidiazonium species that alkylate the 6- and 7-position of guanine in DNA. Substantial evidence suggests that alkylation of DNA at the O6 position of guanine is the cytotoxic lesion induced by alkylating agents such as TEM or its metabolite MTIC (Tisdale, 1987; Lee et al., 1991; Chen et al., 1993; Mitchel and Dolan, 1993). The choice of the triazene TEM as a control drug in this study was inspired by its proven clinical activity in the treatment of solid tumors such as gliomas and malignant melanoma and its significant inactivity in Mer+ cells.

On the other hand, the anilinoquinazolines are a novel class of highly receptor type-specific compounds that inhibit EGFR-related signal transduction by competition at the ATP binding site (Ward et al., 1994). The significant number of structure-activity relationship studies on 4-anilinoquinazolines and pyrido[4]pyrimidines as EGFR TK inhibitors is consistent with the compounds binding to the ATP site of EGFR (Rewcastle et al., 1995, 1997, 1998). Molecular modeling suggests that the N-1 atom (see SMA41, Scheme 2) accepts an H-bond from Met-769, N-3 accepts an H-bond from the side chain of Thr-766 on strand 5 deep in the binding cleft, and the anilino moiety is located in an adjacent hydrophobic pocket. The model suggests that the only positions on the inhibitors where substituents can be altered without affecting binding affinity are the 6- and 7-positions, which are located at the entrance of the binding cleft (Rewcastle et al., 1995). Indeed a variety of compounds with bulky side chains on the 6- and 7-positions were synthesized and found to retain significant binding affinity for the EGFR ATP binding site (Rewcastle et al., 1997). We therefore decided to append the alkyltriazene moiety to the 6-position of the quinazoline heterocycle. Thus, as outlined in Scheme 2, based upon the mechanism of hydrolytically cleavage of 1,2,3-triazenes and the structure-activity relationship of quinazolines, SMA41 was designed to release 1) SMA52, a competitive inhibitor of the ATP binding site of EGFR, and 2) the DNA damaging methylidiazonium species. In addition, this chimeric molecule was designed to retain small enough to be able to interact with the receptor on its own. This property was introduced with the purpose of targeting high EGFR-expressing cells. Interactions of SMA41 with the ATP binding site of EGFR would promote its intracellular retention, thereby favoring more intracellular degradation of this drug.

SMA41 was found to be able to release SMA52 in serum-containing cell culture media and to possess 1) a dual DNA damaging and a cellular phosphotyrosine inhibitory activity, and 2) superior antiproliferative effects compared with its clinical triazene counterpart TEM and its metabolite SMA52 alone. This novel strategy is designated as the “combi-targeting concept”.

Materials and Methods

Drug Treatment. SMA41 and SMA52 were synthesized in our laboratories according to known procedures (Cameron et al., 1985; Manning et al., 1985; Rewcastle et al., 1995). Temozolomide was provided by Schering-Plough Inc. (Kenilworth, NJ). In all assays, drug was dissolved in DMSO and subsequently diluted in sterile RPMI-1640 media containing 10% fetal bovine serum (Life Technologies, Burlington, Canada) immediately before the treatment of cell
The half-life of SMA41 under physiological conditions was studied by UV-spectrophotometry using an Ultraspec 2000 Pharmacia Biotech spectrophotometer. SMA41 was dissolved in a minimum volume of DMSO, diluted with RPMI-1640 medium supplemented with 10% serum, and absorbances read at 340 nm in a UV cell maintained at 37°C with a circulating water bath. The half-life was estimated by a one-phase exponential decay curve-fit method using the GraphPad software package (GraphPad Software, Inc., San Diego, CA).

EGFR Binding Assay. Nunc MaxiSorp 96-well plates were incubated overnight at 37°C with 100 μl/well of 0.25 mg/ml poly(l-glutamic acid-L-tyrosine, 4:1) PGT in PBS. Excess PGT was removed and the plate was washed three times with TWEEN 20 (0.1%) in PBS. The kinase reaction was performed as previously described using 15 ng/well EGFR affinity-purified from A431 cells (Moyer et al., 1997; Vincent et al., 2000) (generous gift from Pfizer Inc., Groton, CT, and commercial supplies from BIOMOL, Gaithersburg, MD). The compound was added and phosphorylation initiated by the addition of ATP. After 8 min at room temperature with constant shaking, the reaction was terminated by aspiration of the reaction mixture and rinsing the plate four times with wash buffer (TWEEN 20 (0.1%) in PBS). Phosphorylated PGT was detected following a 25-min incubation with 50 μl/well of HRP-conjugated PY54 anti-phosphotyrosine antibody diluted to 0.2 μg/ml in blocking buffer (3% bovine serum albumin; 0.05% TWEEN 20). Antibodies were removed by aspiration, and the plate washed four times with wash buffer. The signals were developed by the addition of 50 μl/well of 3,3',5,5'-tetramethylbenzidine peroxidase substrate (Kierkegaard and Perry Laboratories, Gaithersburg, MD) and following blue color development, 50 μl of H2O2 (0.09 M) was added per well, and plates were read at 450 nm using a Bio-Rad ELISA reader (model 2550).

EGF-Induced Autophosphorylation Assay. A431 cells were preincubated in a six-well plate (1 × 10^6) with 0.1% serum at 37°C overnight for 24 h after which they were exposed to a dose range of each drug for 2 h and subsequently treated with 50 ng/ml EGF for 30 min at 37°C. Thereafter, they were washed with PBS and resuspended in cold lysis buffer (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% Nonidet P-40, 1 mM EDTA; 5 mM NaF; 1 mM Na3VO4; protease inhibitor tablet (Roche Biochemicals, Laval, Canada). The lysates were kept on ice for 30 min and collected by centrifugation at 10,000 rpm for 20 min at 4°C. The protein concentrations were determined against a standardized control using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (40 μg/ml from each lysate were added to a 12% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Nonspecific binding on the polyvinylidene difluoride membrane was minimized with a blocking buffer containing nonfat dry milk (3%) in PBS. The membrane was incubated with primary antibodies [either anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) for the detection of phosphotyrosine, or anti-EGFR (Neomarkers, Fremont, CA) for determination of corresponding receptor levels, and anti-β-tubulin (Neomarkers) for the detection of equal loading]. Thereafter, blots were incubated with HRP-goat anti-mouse antibody (1:200 dilution; Bio-Rad Laboratories) and the bands visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Band intensities were measured using the SynGene GeneTools software package.

EGF-Induced Total Cellular Tyrosine Phosphorylation Assay. A431 cells were preincubated in a 96-well plate (1 × 10^6) with 0.1% serum at 37°C overnight. The drugs were added for 15 min in serum-free media, and cells were subsequently given EGF (50 ng/ml) for 30 min at 37°C. Cells were fixed with a 1:1 mixture of methanol and acetic acid for 30 min at 4°C. Nonspecific binding was blocked with bovine serum albumin (1%) in PBS for 1 h at 37°C, after which 0.1 μg/ml HRP-conjugated anti-phosphotyrosine antibody (Upstate Biotechnology) was added in the same buffer for 1 h at room temperature. 3,3',5,5'-Tetramethylbenzidine peroxidase (200 μl) substrate (Kierkegaard and Perry Laboratories) was added to each well.
and following blue color development, \( \text{H}_2\text{SO}_4 \) (0.9 M) was administered to each well, and plates were read at 450 nm.

**Alkaline Comet Assay for Quantification of DNA Damage.** A modified alkaline comet assay technique was used to quantitate DNA damage induced by SMA41, SMA52, and TEM. A431 cells were exposed to drugs for 30 min or 2 h, and harvested with trypsin-EDTA. The cells were subsequently collected by centrifugation and resuspended in PBS. The resulting cell suspension was diluted to approximately \( 10^6 \) cells, and mixed with agarose (1%) in PBS at 37°C in a 1:10 dilution. The gels were cast on Gelbond strips (Mandel Scientific, Guelph, Canada) using gel casting chambers, as previously described (McNamee et al., 2000), and then immediately placed in a lysis buffer [2.5 M NaCl, 0.1 M tetra-sodium EDTA, 10 mM Tris-base, 1% (w/v) N-lauryl sarcosine, 10% (v/v) DMSO, and 1% (v/v) Triton X-100]. After being kept on ice for 30 min, the gels were gently rinsed with distilled water and then immersed in a second lysis buffer (2.5 M NaCl, 0.1 M tetra-sodium EDTA, 10 mM Tris-base), containing 1 mg/ml proteinase K for 60 min at 37°C. Thereafter, the gels were rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37°C, and electrophoresed at 300 mA for 60 min. The gels were subsequently rinsed with distilled water and placed into 1 M ammonium acetate for 30 min. They were further soaked in 100% ethanol for 2 h, dried overnight, and subsequently stained with SYBR Gold (1/10,000 dilution of stock supplied from Molecular Probes, Eugene, OR) for 20 min. For evaluation of comets, DNA damage was assessed using the Tail Moment parameter (i.e., the product of the distance between the barycenters of the head and the tail of the comet). A minimum of 50 cell comets was analyzed for each sample, using ALKOMET version 3.1 software, and values are an average of tail moments for the entire cell population.

**Results**

**Degradation of SMA41**

SMA41 was allowed to degrade in RPMI-1640 supplemented with 10% fetal calf serum at 37°C and its half-life measured by UV-spectrophotometry. It was found to be significantly stable with a \( t_{1/2} \) of approximately 30 min in serum-containing cell culture medium at 37°C. SMA41 decomposed almost exclusively into SMA52, the structure of which was confirmed both by HPLC analysis of independently synthesized SMA52 and by liquid chromatography-mass spectrometry analyses (Fig. 1), which showed a mass \( M + 1 = 251 \) for the chromatogram peak corresponding to its retention time. Quantification of this peak and calculations using standard curves indicated that SMA41 was converted to SMA52 in a yield of approximately 81%.

**Antiproliferative Activity in A431 Cells**

The SRB assay was used to evaluate the antiproliferative activity of different compounds in the human squamous carcinoma of the vulva cell line A431 in which EGFR constitutive activity, as reflected by tyrosine phosphorylation under basal conditions, has been shown to be sensitive to antiproliferative agents targeting the EGFR in vitro or in vivo (Lanzi et al., 1997). In addition, this cell line expresses detectable levels of the DNA repair enzyme MGMT (Pornace et al. 1990). The MGMT status of our A431 cell line was also confirmed by Western blotting using a commercially available anti-MGMT antibody (Pharmingen International, Toronto, Canada) (data not shown). Under 72-h continuous exposure the results, illustrated by Fig. 2a, showed that SMA41 is 1.8-fold more potent (IC\( _{50} \) = 36 \( \mu \)M) than its metabolite SMA52 alone (IC\( _{50} \) = 59 \( \mu \)M, Fig. 2b), and 10-fold more potent than TEM (IC\( _{50} \) = 366 \( \mu \)M, Fig. 4) in the MGMT-proficient cell line A431. A clonogenic assay as illustrated by Fig. 3 showed that the antiproliferative activity of SMA41 was in the same range as that of SMA52 (IC\( _{50} \) SMA41 = 4 \( \mu \)M, IC\( _{50} \) SMA52 = 3.7 \( \mu \)M). However, when the cells were treated for only 2 h and further incubated in drug-free medium, an almost complete loss of activity was observed for SMA52 (IC\( _{50} \) > 100 \( \mu \)M, Fig. 2b), indicating that it induced significantly reversible growth inhibitory activities. In contrast, SMA41 showed significant retention of activity with little change in the IC\( _{50} \) values [IC\( _{50} \) (2 h) = 36 \( \mu \)M, IC\( _{50} \) (72 h) = ~30 \( \mu \)M].

To demonstrate the antiproliferative advantages of combining the EGFR and DNA targeting mechanisms in a single molecule, we studied the combined effect of SMA52 (independently synthesized) with that of TEM using the SRB assay (Fig. 4). Using eq. 1 to determine the nature of interactions between these two drugs, the results showed that the CI\( _{50} \) at the 50% effect for SMA52 + TEM is approximately 0.6, indicating a subadditive interaction. However, under identical conditions the antiproliferative activity of the chimeric SMA41 was 4-fold more pronounced than that of the two-drug combination (IC\( _{50} \) SMA41 = 134 \( \mu \)M).

**Binary Targeting Properties of SMA41**

The significant antiproliferative activity of SMA41 in a methyltriazeno-resistant cell stimulated our interest in further dissecting its binary (EGFR and DNA) targeting properties. This was achieved by two types of assays: EGF-stimulated tyrosine phosphorylation and DNA damage.

**Inhibition of EGFR TK Activity.** In a competitive EGFR binding assay (Fig. 5), SMA41 (IC\( _{50} \) = 0.2 \( \mu \)M) showed a 5-fold stronger binding affinity than SMA52 (1.02 \( \mu \)M) for the ATP site of the purified receptor. TEM did not show any significant affinity for this receptor (IC\( _{50} \) > 100 \( \mu \)M). In an ELISA-based whole cell assay, SMA41 and SMA52 showed comparable levels of inhibition of EGF-induced total cellular phosphorylation (Fig. 6). Similarly, Western blot analysis (Fig. 7) demonstrated that both drugs induced almost equal levels of inhibition of EGF-induced EGFR autophosphorylation (IC\( _{50} \) SMA41 = 8.44 \( \mu \)M, IC\( _{50} \) SMA41 = 12.5 \( \mu \)M). In contrast to SMA41 and SMA52, TEM did not exhibit any EGFR binding affinity, nor did it inhibit EGF-induced auto-

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**Fig. 1.** Partial degradation of SMA41 to SMA52 in RPMI-1640 medium supplemented with 10% serum at 37°C. The peak at around 11 min (1) corresponds to SMA52 and the one at 15 min (2) represents undegraded SMA41. Peaks in 2- to 6-min range are medium components. An almost exclusive decomposition of SMA41 into SMA52 was observed.
phosphorylation in A431 cells (IC$_{50}$ > 100 μM) in the specified dose ranges.

**Quantitation of DNA Damage.** Using the alkaline comet assay, it was demonstrated that, in contrast to SMA52 (Fig. 8c), both SMA41 and TEM were capable of inducing DNA damage in a dose-dependent manner. However, differences were observed in the kinetics of dose-dependent DNA damage induced by SMA41 compared with TEM. For SMA41, the trend was to induce rapid nuclear condensation at the high-
est doses (25–100 μM), leading to a reduction in comet tailing. For SMA41, significant comet tail moment could only be observed in the 6 to 25 μM range after a short 30-min and 2-h drug exposures (Fig. 8a). With a 2-h exposure, a decrease in tail moment was observed at concentrations above 6 μM, concurrent with observable nuclear condensation likely due to a rapid onset of apoptosis. In contrast, TEM (Fig. 8b) exhibited a dose-dependent increase in comet tail moment under 30-min exposure with a remarkable enhancement under the longer 2-h drug exposure (Fig. 8b). This is prima facie evidence that despite being two methylating agents, the mechanisms of action of SMA41 may be markedly different from that of TEM.

Fig. 6. Inhibition of EGF-induced total cellular phosphorylation by SMA41, SMA52, and temozolomide. Total cellular phosphorylation was measured in A431 cells using an immunoassay with anti-phosphotyrosine antibodies. Each point represents at least two independent experiments run in duplicate.

Fig. 7. a, inhibition of EGF-stimulated autophosphorylation by SMA41 in A431 cells. Cells were exposed to the indicated concentrations (μM) of drug for 2 h, after which they were stimulated with 50 ng/ml EGF (lanes 1 and 3–8). A nonstimulated control is shown in lane 2. Cell lysates were prepared following a 30-min exposure to EGF, and phosphotyrosine was detected by Western blotting with anti-β-tubulin used as a loading control. Membranes were stripped of anti-phosphotyrosine and reprobed with anti-EGFR antibodies to detect receptor levels. b, comparison between the inhibition of EGF-stimulated autophosphorylation by SMA41, SMA52, and temozolomide in A431 cells. The film was scanned and the bands were analyzed and quantified using Syngene GeneTools software. Values are percentage of control of phosphotyrosine/β-tubulin.

Fig. 8. Quantitation of DNA damage using the alkaline comet assay. Tail moment was used as a parameter for the detection of DNA damage in A431 cells exposed to SMA41 (a), TEM (b), and SMA52 (c) for 30 min or 2 h.
Discussion

Overexpression of EGFR is common in a wide variety of major human solid tumors of epithelial origin such as breast, colorectal, and head and neck ovarian and bladder carcinomas (Yaish et al., 1988; Lanzi et al., 1997; Modjtahedi and Dean, 1998). EGF binding induces receptor dimerization, autophosphorylation, and activation of mitogenic signaling. The A431 cell line expresses a large number of EGF binding sites and also the high-affinity EGF ligand transforming growth factor TGFα (Lanzi et al., 1997). This translates into aggressive autocrine-controlled growth in vitro. Blocking A431 cell proliferation has become the standard screen for antiproliferative inhibitors of EGFR TK activity (Yaish et al., 1988; Lanzi et al., 1997). This cell line also expresses the alkyltriazene resistance-associated DNA repair enzyme MGMT and is as demonstrated herein (Fig. 2c) resistant to the cyclic 1-methyl-1,2,3-triazene TEM (IC_{50} = 366 μM). Therefore, it represents a good model for the determination of the pharmacological advantages of simultaneous targeting of EGFR and DNA in EGF-expressing refractory tumors.

Dacarbazine and TEM, two drugs of monomethyltriazenes, are the most active drugs in the treatment of malignant melanomas and gliomas (Carter et al., 1976; Hill et al., 1989; Lee et al., 1992; Carter et al., 1994). As outlined in Scheme 1, the cytotoxic monalkyltriazene MTIC degrades under physiological conditions to generate a variety of metabolites, the critical reaction being the heterolysis of the nonconjugated tautomer to generate the arylamine 5-amino metabolites, the critical reaction being the heterolysis of the nonconjugated tautomer to generate the arylamine 5-amino.
results in toto give prima facie evidence that SMA41 is a novel triazene with a significant EGFR tyrosine kinase inhibitory activity, a property that has never been observed before for any class of mono- or dialkyltriazenes.

Based upon known principles of medical oncology, which suggest that rapidly proliferating cells are more sensitive to DNA damaging agents than slow-growing ones, it was feared that the cytostatic effect of EGFR TK inhibition would initially block proliferation and thereby decrease cell sensitivity to the DNA damage associated with the concomitantly generated methylidiazonium species. This would translate into a rather antagonistic effect. To test this hypothesis, we mimicked the combined effect of the two mechanisms of action by designing a two-drug combination model involving SMA52 (an EGFR TK inhibitor) and TEM (a DNA damaging agent). The results showed a subadditive interaction and not an antagonistic one between these two drugs. Moreover, it is noteworthy that SMA41 was more potent than the two-drug combination. This suggests that a single molecule formulated as a masked form of these two types of agents may be more efficacious than a two-drug combination encompassing individual monoalkyltriazenes and EGFR TK inhibitors.

Since SMA41 can both block phosphorylation induced by EGF and damage genomic DNA, its over 8-fold (SRB assay) and over 90-fold (clonogenic assay) greater potency compared with TEM may result from the combined effects of these two distinct mechanisms of antiproliferative activities. The binary targeting may trigger signal transduction associated with the induction of apoptosis. Indeed, in contrast to SMA52 and TEM, significant nuclear condensation was observed in cells treated with SMA41 for 2 h in the 25 to 100 μM range. The effect of binary targeting on the expression and activity of MGMT and the mechanism of apoptosis induced by SMA41 are now being investigated in our laboratory and the results will be reported in due course.

A significant body of evidence has accumulated to suggest that overexpression of EGFR is a marker for poor prognosis in many solid tumors. Selective inhibitors of tyrosine phosphorylation by EGFR are now considered an important class of anticancer drugs and two members of the 4-(phenylamino)quinazoline class are now in clinical trial. Despite the significant EGFR inhibitory activity of these reversible inhibitors, the high intracellular concentrations of ATP is a major barrier to sustained inhibition of EGFR-stimulated signal transduction in tumor cells. More recently, this problem was addressed by Smaill et al. (1999) who showed that quinazolines containing acryloyl function at the 6-position could induce irreversible inhibition of EGFR by alkylating cysteine 773 of the enzyme. A recently synthesized water-soluble analog of this class has now been selected for phase I clinical trial (Jeff et al., 2000). It is noteworthy that despite being irreversible inhibitor of EGFR, when apoptosis is not triggered, if the cells respond to alternative growth hormones (e.g., heregulin or platelet-derived growth factor) these compounds may still not induce a sustained growth inhibitory activity. Our novel SMA41 presents the advantage of being not only capable of blocking EGF-stimulated signal transduction on its own but also generating a DNA alkylating species that may inflict irreversible cytotoxic DNA lesions. Moreover, this compound was designed to release another intact EGFR TK inhibitory molecule (e.g., SMA62) that may further enhance its growth inhibitory activity. Our results showed that these combined properties conferred increased potency to a monoalkyltriazene against an MGMT-proficient tumor cell line with marked resistance to the clinical drug TEM (IC50 = 366 μM).

Also, the current study, which was primarily designed to identify the principal targets of SMA41, has conclusively demonstrated that this one-molecule combination showed superior activity compared with a two-drug combination involving TEM + SMA52. Further studies are now ongoing to characterize the effects of SMA41 on growth stimulation by a wide variety of hormones, including EGF, transforming growth factor, platelet-derived growth factor, and insulin before the demonstration of in vivo efficacy of this novel approach termed the combi-targeting concept.

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